Up-regulation of the Pit-2 Phosphate Transporter/Retrovirus Receptor by Protein Kinase C ε*

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The membrane receptors for the gibbon ape leukemia retrovirus and the amphotropic murine retrovirus serve normal cellular functions as sodium-dependent phosphate transporters (Pit-1 and Pit-2, respectively). Our earlier studies established that activation of protein kinase C (PKC) by treatment of cells with phorbol 12-myristate 13-acetate (PMA) enhanced sodium-dependent phosphate (Na/P_i) uptake. Studies now have been carried out to determine which type of Na/P_i transporter (Pit-1 or Pit-2) is regulated by PKC and which PKC isotypes are involved in the up-regulation of Na/P_i uptake by the Na/P_i transporter/viral receptor. It was found that the activation of short term (2-min) Na/P_i uptake by PMA is abolished when cells are infected with amphotropic murine retrovirus (binds Pit-2 receptor) but not with gibbon ape leukemia retrovirus (binds Pit-1 receptor), indicating that Pit-2 is the form of Na/P_i transporter/viral receptor regulated by PKC. The PKC-mediated activation of Pit-2 was blocked by pretreating cells with the pan-PKC inhibitor bisindolylmaleimide but not with the conventional PKC isotype inhibitor Gö 6976, suggesting that a novel PKC isotype is required to regulate Pit-2. Overexpression of PKCε, but not of PKCα, -δ, or -ζ, was found to mimic the activation of Na/P_i uptake. To further establish that PKCε is involved in the regulation of Pit-2, cells were treated with PKCε-selective antisense oligonucleotides. Treatment with PKCε antisense oligonucleotides decreased the PMA-induced activation of Na/P_i uptake. These results indicate that PMA-induced stimulation of Na/P_i uptake by Pit-2 is specifically mediated through activation of PKCε.

Protein kinases, including members of the protein kinase C family, regulate numerous biological functions, including intracellular protein trafficking and the activities of different ion transporters (1). Previously, we showed that sodium-dependent phosphate (Na/P_i)[1] transport was stimulated by protein kinase C (PKC) and inhibited by protein kinase A in NIH 3T3 cells.

Phorbol 12-myristate 13-acetate (PMA), an activator of PKC, was found to cause a rapid (within 10 min) stimulation of short term Na/P_i uptake (2). However, at that time the identity of the Na/P_i transporter stimulated in response to PMA was not known, and this prevented further characterization of the PKC-mediated activation of the transporter. More recently, cell surface receptors for the gibbon ape leukemia virus (Glvr-l and Pit-1) and the amphotropic murine leukemia virus (Ram-1, Ear, and Pit-2) were demonstrated to serve as Na/P_i transporters in the normal cellular physiology of diverse cell types (3–5).

Amino acid sequence data obtained for the Pit-1 and Pit-2 receptor-transporters has revealed multiple sites potentially susceptible to phosphorylation by protein kinases, including PKC, which are found within the hydrophilic cytoplasmic domain of both transporters between residues 250 and 450. Indeed, parathyroid hormone-induced regulation of transporter function mediated through activation of protein kinase A and PKC has been described for type I and II Na/P_i transporters present in kidney brush-border membranes (6). In addition to parathyroid hormone, prostaglandin E2, insulin-like growth factor 1, and vitamin D_3 all have been reported to regulate Na/P_i uptake through activation of PKC in osteoblasts, another cell type that uses high levels of inorganic phosphate (7–10). It also has been suggested that a phospholipase Cγ-PKC signaling pathway is responsible for the up-regulated P_i transport observed with platelet-derived growth factor treatment of osteoblast-like cells. (11–13). In osteoblasts it is possible that the more ubiquitously expressed type III Na/P_i transporters, such as Pit-1 and Pit-2, might be involved in the PKC-regulated uptake of P_i. Conversely, activation of PKC has been reported to inhibit P_i uptake in opossum kidney cells, which may indicate different regulation of the renal type I and II transporters (6, 14, 15). These differences in PKC-mediated regulation of P_i uptake may be a consequence of different expression patterns of either PKC and/or Na/P_i transporter isotypes in different cell lines.

One of the major difficulties in determining which PKC isotypes are involved in regulating Na/P_i transport is that different cell types express various combinations of PKC isoforms. Protein kinase C is a family of at least 11 serine- and threonine-specific phosphotransferase isoenzymes that are characterized by a high degree of homology in their catalytic and cysteine-rich domains (1). Although the possible role(s) of different PKC isozymes in cell growth and differentiation has been well studied (for review, see Ref. 1), much less is known of their potential involvement in modulating intracellular trafficking of transmembrane receptors and up-regulation of ion transporters. In

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[1] The abbreviations used are: Na/P_i, sodium-dependent phosphate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco's modified Eagle's medium; A-MuLV, amphotropic murine retrovirus; E-MuLV, ecotropic MuLV; GALV, gibbon ape leukemia retrovirus; AON, antisense oligonucleotide; pε-MTH, pε-metallothionein.

FIG. 1. **Effects of infection of cells with the amphotropic murine leukemia virus, the ecotropic murine leukemia virus, and the gibbon ape leukemia virus on the activation of sodiumdependent P_i uptake by PMA.** NIH 3T3 cells were productively infected with wild type A-MuLV (*A*, ○) and E-MuLV (*B*, ○) and then treated with 1 μM PMA for the periods indicated. Mink fibroblasts were infected with wild type A-MuLV (*C*, ○) and wild type GALV-1 (*D*, ○) and then treated with 1 μM PMA for the times indicated. Uninfected cells were used as control for each experiment (●). P_i transport activity was determined as described under "Experimental Procedures." The data are given as the mean ± S.E. of two to four separate experiments each carried out in duplicate.
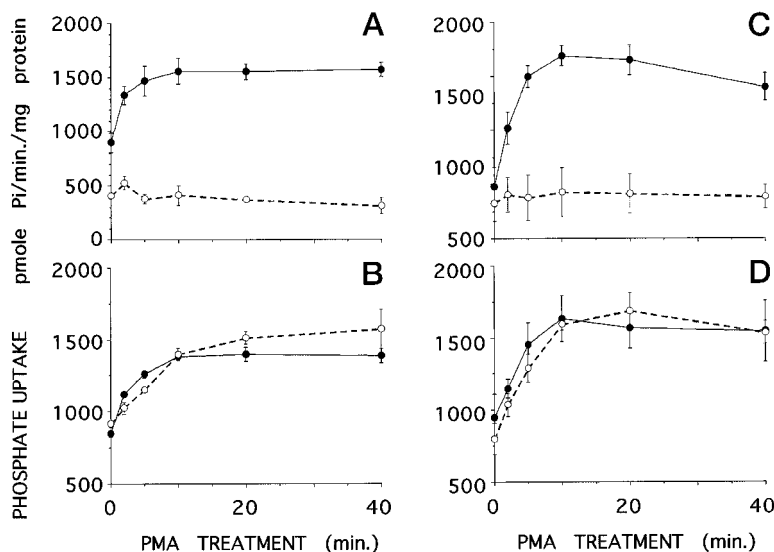
this study we have sought to determine which type of Na/P_i transporter/viral receptor is regulated by PMA activation of PKC and which PKC isotype(s) may be involved in the up-regulation of Na/P_i uptake in NIH 3T3 cells. The results presented in this communication indicate that the Pit-2 Na/P_i transporter/viral receptor is specifically activated by PMA stimulation of PKCε.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were purchased from Biofluids Inc. (Rockville, MD). PMA, bisindolylmaleimide, and Gö 6976 were products of Calbiochem (San Diego, CA). ^{32}P-Labeled monopotassium phosphate was from ICN (Costa Mesa, CA). The PKC isotype-specific antisense and scrambled oligonucleotides (ISIS 17260, PKCε antisense; ISIS 17261, PKCε scrambled control) were from ISIS Pharmaceuticals. PKCε isotype-specific polyclonal antibodies were purchased from Life Technologies, Inc.; PKCα-specific monoclonal antibodies were from Upstate Biotechnology (Lake Placid, NY); and PKCδ- and PKCζ-specific monoclonal antibodies were from Transduction Laboratories (Lexington, KY).

Cell Culture—Retrovirus-infected and vector-transduced NIH 3T3 cells were cultured in DMEM supplemented with 10% fetal calf serum. After the cells reached confluency, the medium was changed to serum-free DMEM for 24 h. To induce overexpression of any ectopic gene products, the cells were incubated in the presence or absence of 20 μM zinc acetate, as indicated, to induce the up-regulation of the metallothionein promoter of the pεMTH vector (16).

Generation of Overexpressor Cell Lines—The construction of expression vectors and establishment of PKC overproducer cell lines were carried out as described previously (17). The PKCα, -δ, -ε, and -ζ plasmid constructs were prepared in the pεMTH vector, and overexpressor cell lines were established following protocols described elsewhere (18). Individually picked colonies (10 from each transfection) were selected and combined for further studies to eliminate potential cloning artifacts. These mixed populations of overexpressor cells were used only through 12–14 passages in culture to negate possible outgrowth of one particular clone.

Phosphate Uptake Measurement—Sodium-dependent phosphate uptake was determined as described previously (2).

Retrovirus Infections—NIH 3T3 murine fibroblasts and mink lung fibroblasts (ATCC CCL 64) were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum. NIH 3T3 cells were infected with wild type amphotropic murine retrovirus (A-MuLV) strain 4070A or the 57A Friend strain of ecotropic MuLV (E-MuLV). Mink fibroblasts expressing gibbon ape leukemia retrovirus (GALV)-competent PiT-1 were infected with wild type A-MuLV strain 4070A or infected with a GALV strain (SEATO), as described previously (4). Productive infection was monitored by measuring the reverse transcriptase activity found in the cell media of the infected cells (19).

Antisense Oligonucleotide Treatment—NIH 3T3 fibroblasts were cultured in 150-mm tissue culture dishes until they reached ~80% confluency. The cells then were harvested by trypsinization, washed with

DMEM, and resuspended in 400 μl of cytosalt electroporation buffer (75% cytosalts (120 mM KCl, 0.15 mM CaC1$_2$, 10 mM K$_2$HP0$_4$, pH 7.6, 6.5 mM MgCl$_2$) and 25% Opti-MEM I). Twenty-μl aliquots of PKC isotype-specific or scrambled control oligonucleotides were added to the cells resuspended in prechilled BTX disposable electroporation cuvettes (P/N 640; 4-mm gap) to reach the indicated concentrations and then incubated on ice for 5 min. The oligonucleotides indicated were introduced into the cells by electroporation with a BTX Electro Square Porator (settings: low voltage mode, 99 msec; charge voltage, 475 V; pulse length, 1 msec; number of pulses, 4). The electroporated cells were kept at room temperature for 10 min and then seeded onto 100-mm tissue culture plates for immunoblot studies and onto 24-well plates for P_i uptake measurements. Western blot analysis of PKC isotypes and P_i uptake studies were carried out 24 h after introduction of the antisense oligonucleotides.

Western Blot Analysis—Proteins were separated by precast 4–20% SDS-polyacrylamide gel (Owl Separation Systems, Portsmouth, NH) electrophoresis and electrophoretically transferred from the gel onto Protran membranes (Schleicher & Schuell, Keene, NH), and immunoreactive proteins were detected as described elsewhere (16, 18).

RESULTS AND DISCUSSION

Most retroviruses have been found to use distinct cell surface receptors for specific cellular recognition and infection (for review, see Ref. 20). Furthermore, studies have revealed that the normal cellular function of a number of these viral receptors is to serve as membrane transport proteins (20). NIH 3T3 cells have been found to express several of these viral receptor/transporters, including Pit-1, Pit-2, and the cationic amino acid transporter CAT/y+, as determined by viral infection studies and reverse transcription-polymerase chain reaction analysis. NIH 3T3 cells are susceptible to infection by A-MuLV via Pit-2 and to infection with E-MuLV via the CAT/y+ amino acid transporter. However, because of the presence of specific point mutations in the endogenous murine Pit-1, Pit-1 is not functional as a GALV receptor in NIH 3T3 cells.

Effect of Viral Infection on PMA-induced Activation of Phosphate Transport—Previous studies have established that infection of cells with retroviruses that selectively recognize either Pit-1 or Pit-2 resulted in the specific down-modulation of phosphate uptake mediated by that receptor/transporter (3, 5). A similar phenomenon has been observed to occur with the E-MuLV CAT/y+ receptor (21, 22). To examine which of the viral receptor/P_i transporters are subject to regulation by PMA activation of PKC, studies were carried out with NIH 3T3 cells infected with different C-type retroviruses. The results presented in Fig. 1*A* demonstrate that infection with A-MuLV decreased short term (2-min) basal Na/P_i transport (from 900 ±

91 to 404 ± 28 pmol of P_i/min/mg of protein) and, importantly, completely abolished the activation of P_i transport noted with exposure of cells to PMA. Infection of cells with E-MuLV would not be expected to alter P_i uptake, because this virus binds to a distinct cell surface cationic amino acid transporter (21, 22). Thus, to control for possible pleiotropic effects of retrovirus infection on Na/P_i transport, P_i uptake experiments were carried out with NIH 3T3 cells infected with E-MuLV (Fig. 1*B*). The results indicated that infection with E-MuLV did not alter either basal or PMA-induced Na/P_i transport.

As noted, GALV binds to the Pit-1 receptor of mink fibroblasts and selectively down-modulates P_i uptake caused by Pit-1 (4). To determine whether Na/P_i transport mediated by Pit-1 or Pit-2 is stimulated by activation of PKC, P_i uptake was measured in mink cells infected with either A-MuLV or GALV. Infection of these cells with A-MuLV again completely abolished the activation of P_i transport noted with exposure of cells to PMA (Fig. 1*C*). Although GALV infection did result in an ~25% decrease in basal Na/P_i transport, which apparently is attributable to down-modulation of Pit-1, it did not decrease the PMA-induced stimulation of P_i uptake noted in these cells (Fig. 1*D*). Taken together, these results obtained with C-type retrovirus-infected cells indicate that Pit-2 is the P_i transporter/viral receptor that is up-regulated with exposure of cells to PMA.

Effect of Selective PKC Inhibitors on PMA-induced Up-regulation of Pit-2 P_i Transport—Although studies have established a general role for PKC in the regulation of several membrane transport mechanisms, little is known concerning the functional role(s) of specific PKC isotypes in these regulatory processes. Thus, studies were initiated to determine which PKC isotype(s) are involved in mediating the PMA-induced up-regulation of the Pit-2 transporter/receptor in NIH 3T3 cells. NIH 3T3 cells express a limited, but representative, set of different PKC isozymes (PKCα, PKCδ, PKCε, and PKCζ) (18, 23). Because PMA does not directly activate atypical PKCζ, this isotype would not appear to be involved in the short term PMA-induced activation of Pit-2 P_i transport. Thus, experiments were initiated to address which class of PKC isoform stimulates P_i transport in response to activation by PMA. Two different PKC inhibitors were used to determine whether the PMA-induced activation of Na/P_i transport in NIH 3T3 cells was mediated via a conventional, Ca^{2+}-dependent (PKCα), or a novel, Ca^{2+}-independent (PKCδ and PKCε), isotype. Addition of the pan-specific bisindolylmaleimide inhibitor, which inhibits both classical and novel isotypes, resulted in pronounced inhibition of the PMA-induced activation of Na/P_i uptake (Fig. 2). Conversely, treatment of the cells with the Gö 6976 PKC inhibitor, which selectively inhibits only the classical PKC isotypes, did not cause significant inhibition of the PMA-induced up-regulation of P_i transport. These results suggested that the classical PKCα isoform likely was not involved in mediating the activation of Na/P_i uptake by Pit-2 with exposure of NIH 3T3 cells to PMA.

Effect of Overexpression of PKC Isotypes on PMA-induced Stimulation of Pit-2 Na/P_i Uptake—To further resolve which of the PKC isotype(s) may be involved in mediating the PMA activation of the Na/P_i uptake, we used NIH 3T3 cells overexpressing PKCα, -δ, -ε, or -ζ isozymes to determine the ability of each isotype to enhance Pit-2 P_i transport activity in the absence of PMA treatment. The cell cultures were shifted to serum-free media and incubated in the presence of 20 μM zinc acetate for 24 h to enhance expression of the indicated PKC isotype directed by the metallothionein promoter of the pε-MTH vector. Overexpression of PKCε was found to increase Na/P_i uptake, whereas overexpression of PKCα, PKCδ, and
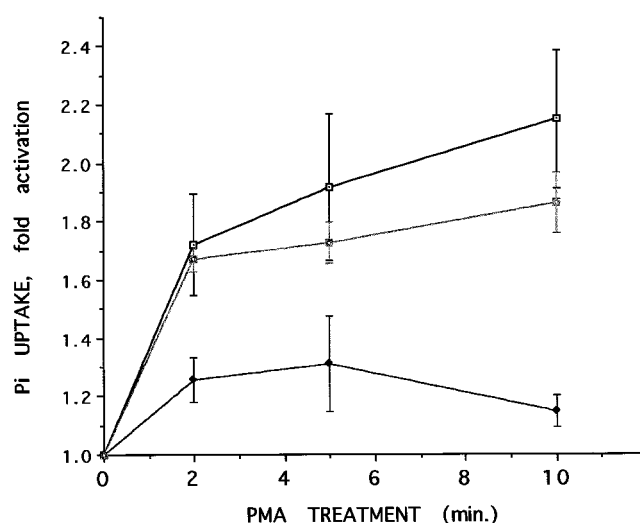

FIG. 2. **Effect of PKC inhibitors on the PMA-induced stimulation of P_i uptake.** Serum-starved NIH 3T3 cells were preincubated with 0.01% dimethylsulfoxide as solvent control (□), 500 nM bisindolylmaleimide (◆), or 500 nM Gö 6976 (■) for 4 h, and then treated with 1 μM PMA for the times indicated. P_i transport activity was determined as described under "Experimental Procedures." Data represent the mean ± S.E. of three independent experiments performed in duplicate ($n = 6$). As determined by Student's t test, the inhibitory effects noted with bisindolylmaleimide were statistically significant ($p < 0.05$), whereas the slight effects noted with Gö 6976 ($p > 0.4$) were not significant.

PKCζ did not appreciably alter the level of P_i transport relative to the level determined in vector control cells (Fig. 3*A*). Exposure of the PKCε overexpressor cells to 1 μM PMA resulted in only an additional 15–20% increase in P_i transport.[2] Western blot analysis showed that the level of expression was similar for each of the ε epitope-tagged PKC isotypes (Fig. 3*B*). These results indicate that the selective overexpression of PKCε alone can mimic the stimulation of Na/P_i uptake observed with PMA treatment of wild type cells.

Effect of PKCε-specific Antisense Oligonucleotide on PMA-induced Stimulation of Pit-2 P_i Transport—To further support the findings that PKCε is the isotype involved in mediating the PMA-induced activation of the Pit-2 P_i transporter, studies were carried out with PKCε-selective antisense oligonucleotide (AON) to specifically inhibit PKC-ε in the cell. As shown in Fig. 4*A*, pretreatment of NIH 3T3 cells with increasing concentrations of PKCε-AON significantly decreased the expressed levels of PKCε and had no effect on the levels of PKCα, PKCδ, or PKCζ. Densitometric scanning to quantitate the intensity of the PKCε bands of cells treated with PKCε-AON relative to AON scrambled controls indicated relative band densities of 1.0, 0.59, and 0.28 with 0.24, 1.2, and 2.4 mM oligonucleotide treatment, respectively. Importantly, treatment of the cells with PKCε-AON did result in inhibition of PMA-induced up-regulation of P_i transport (Fig. 4*B*). Treatment of cells with scrambled oligonucleotides did not have any effect on either the intracellular level of PKCε or PMA activation of Na/P_i uptake. Similar experiments with PKCδ-AON (ISIS 17254) caused a significant decrease in the levels of PKCδ but had no effect on PMA-induced stimulation of P_i uptake (data not shown). These data provide additional evidence to support the exclusive involvement of PKCε in mediating PMA activation of Pit-2.

The suggestion that different PKC isotypes play distinct functional roles in the cell by phosphorylating either isoform- or subcellular compartment-specific substrates is widely accepted. However, few studies have been reported that establish

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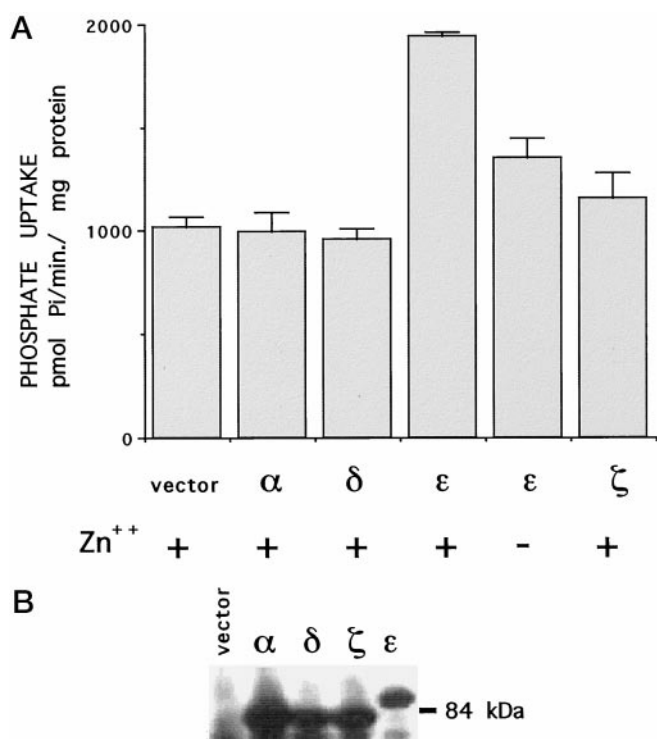[2] Z. Jobbagy, M. V. Eiden, and W. B. Anderson, unpublished results.

FIG. 3. **Effect of overexpression of different PKC isotypes on the PMA-induced stimulation of P$_i$ uptake.** *A*, vector control and PKC isotype overexpressor NIH 3T3 cells were serum starved for 24 h in the presence (+) or absence (−) of 20 μM zinc acetate. Short term (2-min) sodium-dependent P$_i$ transport activity was determined in control and overexpressor cells as described under "Experimental Procedures." Each column represents the mean ± S.E. of three independent experiments performed in duplicate. *B*, Western blot analysis of the levels of ε epitope-tagged PKC isotypes in total cell extracts of the indicated PKC isotype overexpressor. NIH 3T3 cells were serum starved for 24 h in the presence of 20 μM zinc acetate, and total cell extracts then were prepared. Electrophoresis of total 20-μg protein lysate was carried out on 4–20% SDS-polyacrylamide gels, and immunoblot analysis was carried out with anti-ε epitope tag antibody as described under "Experimental Procedures."

FIG. 4. **Effect of PKCε-specific antisense oligonucleotide treatment of NIH 3T3 cells on PMA-induced stimulation of Na/P$_i$-uptake.** The antisense (*a*) and scrambled control (*c*) oligonucleotides at the concentrations indicated were introduced into NIH 3T3 cells by electroporation, and the electroporated cells then were incubated for 24 h in the presence of the introduced oligonucleotides. *A*, selective decrease in PKCε protein levels with treatment of cells with PKCε-specific antisense oligonucleotides. Cells treated with the indicated concentrations of PKCε-specific antisense (*a*, ISIS 17260) or scrambled control (*c*, ISIS 17261) oligonucleotides for 24 h were harvested by scraping into lysis buffer. Aliquots of the cell lysates containing 100 μg of total protein were analyzed for changes in the levels of specific PKC isoforms by Western blotting using PKCε, -α, -δ, and -ζ izotype-specific antibodies. Western blot data represent a characteristic expression pattern of three similar experiments. *B*, treatment of NIH 3T3 cells with PKCε-specific antisense oligonucleotides inhibited the PMA-induced increase in P$_i$ uptake. Cells treated with the indicated concentrations of oligonucleotides for 24 h were exposed to 0.01% dimethylsulfoxide (solvent control) or 1 μM PMA for 10 min, and short term P$_i$ transport activity then was determined as described. Data are presented as the fold increase in P$_i$ uptake in response to PMA treatment above basal values determined in the presence of dimethylsulfoxide. Results are given as the average ± S.E. of three separate uptake experiments performed in duplicate.

that a specific PKC isotype may selectively regulate a given biological function. Although a role for PKC has been implicated in the regulation of numerous membrane transport mechanisms (10, 24–27), little information is available on the specific PKC isotype(s) involved in the regulation of these transport activities. Karim *et al.* (28) attributed the modulation of the Na/H antiport to both PKCα and PKCε. PKCε also has been implicated in the stimulation of anionic amino acid transport (29), and treatment with antisense oligonucleotides to PKCδ has been shown to block the α1-adrenergic activation of Na-K-2Cl cotransport (30). The evidence reported here indicates that PKCε is involved in mediating the PMA-induced up-regulation of Na/P$_i$ uptake by the Pit-2 transporter/viral receptor.

The Pit-1 and Pit-2 viral receptor/P$_i$ transporters share 56% amino acid identity (31). Hydropathy analysis of Pit-1 and Pit-2 suggested the presence of at least two clusters of putative transmembrane-spanning sequences, along with a large intracellular hydrophilic domain located between the sixth and seventh transmembrane helices (4, 19). There are a number of consensus phosphorylation sites in both Pit-1 and Pit-2, particularly within the hydrophilic loop domain. Thus, it is likely that PKCε may directly phosphorylate Pit-2 to stimulate Na/P$_i$ uptake.

However, another mechanism of regulation found with other transporters is induced redistribution of the transporter from intracellular stores to the plasma membrane. For example,
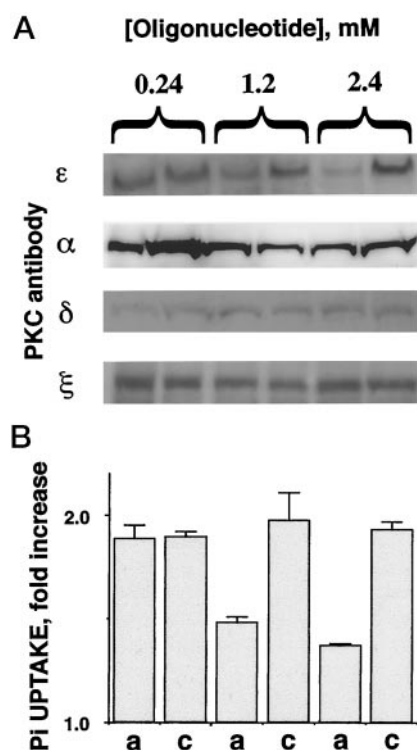
insulin has been reported to regulate the intracellular trafficking of glucose transporter 4 (32). Previously, we have shown that PKCε can regulate Golgi-related functions, including protein trafficking and secretion (17). To address this possibility, studies were carried out to determine whether PMA still was able to enhance P$_i$ uptake under conditions in which vesicle trafficking from the Golgi to the plasma membrane was blocked by incubating cells at room temperature and by treatment of cells with nocodazole (to disrupt microtubules) and cytochalasin D (to disrupt actin filaments). It was found that these treatments did not block PMA-induced activation of P$_i$ uptake.[2] Although these results indicate that PKCε does not act by modulating the trafficking of PiT-2 from the Golgi to the plasma membrane, they do not fully rule out the possibility that PKCε might act to mediate the rapid recruitment (fusion) of an existing pool of Pit-2-containing vesicles to the plasma membrane.

In addition to their role as representative members of an important family of phosphate transporters, Pit-1 and Pit-2 are

of particular interest as the cell surface receptors for the GALV and A-MuLV retroviruses, respectively. Many current gene therapy protocols use GALV- or A-MuLV-enveloped vectors (33, 34). A basic knowledge of murine leukemia virus receptor regulation and trafficking is likely to be useful in the development and improvement of gene therapy protocols based on the use of these retroviral vectors. Although our results clearly indicate that PKCε is involved in regulating Na/P$_i$ uptake by Pit-2, it remains to be determined whether PKCε-mediated regulation of the Pit-2 transporter/viral receptor might influence recognition of the viral envelope protein and viral entry into the cell.

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